FEBS Letters 580 (2006) 1597–1600

Cyclophosphamide enhances TNF-α-induced apoptotic cell death in murine vascular endothelial cell

Toshio Ohtani[a,*], Tomoyuki Nakamura[a], Ken-ichi Toda[b], Fukumi Furukawa[a]

[a] *Department of Dermatology, Wakayama Medical University, 811-1 Kimi-idera, Wakayama, Japan*
[b] *Department of Dermatology, Kitano Hospital, Osaka, Japan*

Received 4 November 2005; accepted 30 January 2006

Available online 7 February 2006

Edited by Lukas Huber

Abstract Cyclophosphamide (CPA) is one of the therapeutic agents for systemic inflammatory disorders. In murine dermal endothelial cells (F-2), 4-hydroxycyclophosphamide (4-HC), which is active metabolite of CPA, enhanced TNF-α-induced DNA fragmentation. In addition, 4-HC was shown to elevate TNF-α-induced caspase-3 activation. Caspase-8 activation was identified by the treatment of TNF-α, whereas 4-HC was no effect. In contrast, only when treated with 4-HC, caspase-9 activation and the increase in the intracellular expression of Bax were detected. These results suggest that CPA may sensitize endothelial cells to TNF-α-induced apoptosis through a mitochondria-dependent pathway and clinically may contribute to the limitation of inflammatory process.

Keywords: Cyclophosphamide; TNFα; Endothelial cell; Caspase; Bax

1. Introduction

Apoptosis is an essential event for tissue homeostasis and it is tightly regulated at the molecular level. Two main pathways to apoptotic cells have been identified; the one is the interaction of a death receptor and the other is the participation of mitochondria. Pro-apoptotic and anti-apoptotic members of the Bcl-2 family regulate mitochondrial pathway. Either pathway results in the activation of caspase and the cleavage of specific cellular substrate [1].

Endothelial cells are activated by cytokines, such as TNF-α, and play a key role in the inflammatory process of various immunomediated disease [2]. So, the regulation of activated endothelial cells by some agent may lead to prevent inflammatory reactions.

Cyclophosphamide (CPA), which is a widely used anti-cancer prodrug, is effective for autoimmune diseases and systemic vasculitis [3]. It has been shown that CPA suppresses B-cell function with decreased antibody production [4]. However, the mechanism of action to endothelial cell is still not elucidated fully. In the present study, we investigated whether 4-hydroxycyclophosphamide (4-HC), which is active metabolite of CPA, might evoke apoptosis in dermal endothelial cells. We could demonstrate that 4-HC, at therapeutic concentrations, induced apoptosis, and enhanced TNF-α-induced apoptosis. Our data suggested that, at least in part, the anti-inflammatory effects of CPA were mediated by the induction of apoptosis of endothelial cells.

2. Materials and methods

2.1. Cell culture and reagents

The murine dermal endothelial cell clone (F-2) was previously established [5]. PAM 212 and NIH-3T3 were kindly gifts from Dr. Norihisa Matsuyoshi (Kyoto University, Kyoto, Japan). These cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS) at 37 °C under 5% CO_2 in air. 4-Hydroxycyclophosphamide (4-HC) was generously provided by Shionogi Seiyaku Co. Ltd. (Tokyo, Japan). Recombinant murine TNF-α was purchased from R&D Systems (Minneapolis, MN, USA). [4-{3-(4-lodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio}-1,3 benzene disulfonate] (WST-1) was obtained from Roche (Tokyo, Japan). Poly [ADP-ribose] polymerase (PARP) antibody was from Sigma (Tokyo, Japan). Polyclonal rabbit anti-mouse Bax was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The caspase inhibitors Z-DEVD-FMK were from MBL (Nagoya, Japan).

2.2. Cell viability assay

Cells (0.5×10^4 cells per well) were grown in 96-well plates (Falcon Labware, oxnard, CA, USA) with 4-HC at 10 μM or the same amount of distilled water as vehicle. After 24 h, WST-1 was added to each well. Then, cells were incubated for 1 h and absorbance was measured with a microplate reader (Bio-Rad, Tokyo, Japan) at 450 nm, with a reference wavelength of 630 nm. All assays were run in triplicate.

2.3. DNA agarose gel electrophoresis

Cells (1×10^5 cells) were incubated in 10 cm dishes with 4-HC (10 μM) or vehicle. After 24 h, the genomic DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN, Tokyo, Japan) and was electrophoresed on 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

2.4. Sandwich ELISA for histone-associated DNA fragments

Cell death was assessed using a cell death detection kit (Roche). Cells were incubated for 2 h in 96-well plate in the presence or absence of TNF-α (50 ng/ml) and then cultured with 4-HC (10 μM) or vehicle for 24 h. The cells were harvested in lysis buffer and the cytoplasmic and nuclear fractions were separated by centrifugation at $200 \times g$. The supernatant (cytoplasmic) fraction was added to streptavidine-coated microtiter plate, followed by biotin-labeled anti-histone antibody and HRP-conjugated anti-DNA antibody. After 2 h, immune complex was detected using substrate (2,2″-azino-di[3-ethyl-benzthiazolin-sulfonate]) for 10 min and absorbance was measured at 405 nm, with a reference wavelength of 490 nm.

*Corresponding author. Fax: +81 73 448 1908.
E-mail address: tossan@wakayama-med.ac.jp (T. Ohtani).

0014-5793/$32.00
doi:10.1016/j.febslet.2006.01.092

2.5. Assay for activity of caspase-3, caspase-8, and caspase-9

Cytosolic extracts were prepared and assayed for caspase activities according to the manufacture's protocol (MBL). In brief, cells were collected and lysed in an ice-cold lysis buffer. The cell lysate was centrifuged $10000 \times g$ for 1 min at 4 °C and the supernatant fraction was used. The protein concentration was determined using protein assay ESL (Roche). Aliquots of 100 μg of protein were incubated with 0.2 mM DEVD-pNA, IETD-pNA, or LEHD-pNA substrate for 2 h at 37 °C in reaction buffer. The reaction was measured with spectrophotometer (Hitachi, Tokyo, Japan) at a wavelength of 405 nm. Fold increase was calculated by comparing with control.

2.6. Caspase inhibitory assay

Cells were treated with Z-DEVD-FMK for 2 h at 10 μM. Dimethyl sulfoxide (DMSO) was used as a diluent control. Then, cells were incubated for 2 h in 96-well plate in the presence or absence of TNF-α (50 ng/ml) and then cultured with 4-HC (10 μM) or vehicle for 24 h.

2.7. Western blot analysis

Cells were harvested in an ice-cold lysis buffer containing 20 mM HEPES (pH7.4), 2 mM EDTA, 250 mM NaCl, 0.1% Nonident P-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSF, 0.5 μg/ml benzamidine, 1 mM DTT. Insoluble material was removed by $10000 \times g$ for 5 min at 4 °C. Lysates were boiled in sample buffer for 5 min. Samples containing equal amounts of protein (50 μg) were separated by 10% SDS–PAGE and transferred to nitrocellulose membrane. The membrane was blocked for 1 h with 5% non-fat dried milk in TBS containing 0.1% Tween 20 (TBS-T). After incubation for 1 h with anti-PARP antibody or anti-Bax antibody diluted 1:5000 in TBS-T, membranes were washed three times with TBS-T. The secondary antibody was incubated at 1:20000 dilution for 1 h. Following three washes with TBS-T, the protein bands were visualized with ECL system (Amersham Pharmacia Biotech).

2.8. Statistical analysis

Each experiment was performed at least three times and statistical analysis was performed using the two-tailed Students' *t* test.

3. Results

3.1. 4-Hydroxycyclophosphamide (4-HC) induced apoptotic cell death in F-2 cells

The growth inhibitory effect of 4-HC was investigated by WST-1 assay. As shown in Fig. 1, 4-HC at concentration of 10 μM was able to decrease murine endothelial cell (F-2) viability. In keratinocyte (PAM212) and fibroblast (NIH-3T3), 4-HC was no effect at the same concentration. To determine whether 4-HC can induce endothelial apoptosis, DNA ladder assay was performed. DNA laddering was observed in cells treated with 4-HC at concentration of 10 μM (Fig. 2).

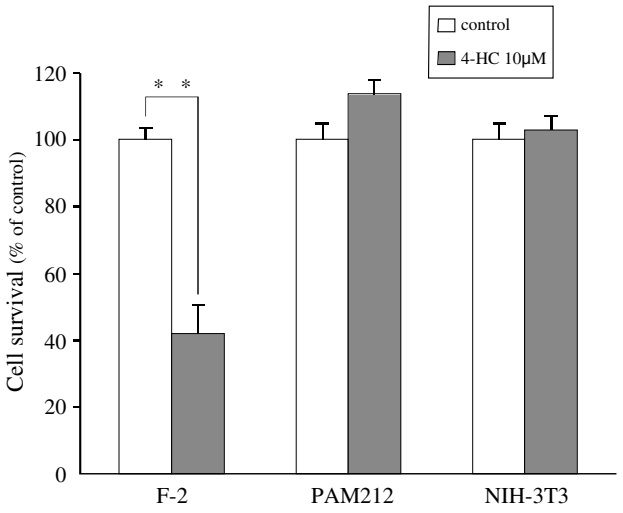

Fig. 1. Effects of 4-HC on cell survival in F-2, PAM212, and NIH-3T3. F-2, PAM212, and NIH-3T3 were grown with 4-HC (10 μM) or vehicle for 24 h and the number of viable cells was estimated by WST-1 assay. The data are presented as means ± S.D. from three independent experiments. $^{**}P < 0.01$.

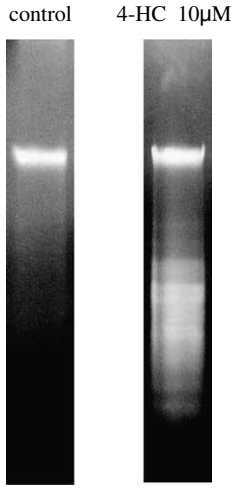

Fig. 2. DNA ladder formation in F-2 treated with 4-HC (10 μM). DNA was fractionated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

3.2. 4-HC enhanced TNF-α-induced DNA fragmentation, caspase-3 activation, and cleavage of PARP

The sandwich ELISA that can detect the enrichment of mono- or oligo-nucleosomes in cytoplasms of apoptotic cells was carried out for the purpose of measuring apoptotic cell death quantitatively. The stimulation with TNF-α (50 ng/ml) or 4-HC (10 μM) showed higher optical absorbance independently in comparison with no stimulation, whereas TNF-α (50 ng/ml) and 4-HC (10 μM) have additional effect (Fig. 3). Next, we examined whether caspase-3 contributed to the apoptosis. While DEVDase activation was induced by TNF-α (50 ng/ml) alone, it was enhanced by the combination of 4-HC (10 μM) and TNF-α (50 ng/ml) (Table 1). In addition, we measured the cleavage of PARP which is a caspase-3 substrate by Western blot analysis. The expression of the cleaved subunit of PARP was upregulated in the cells treated with the combination of TNF-α (50 ng/ml) and 4-HC (10 μM) compared with TNF-α (50 ng/ml) alone (Fig. 4).

3.3. Caspase-3 inhibitor attenuated TNF-α and/or 4-HC-induced apoptosis

To further investigate the involvement of caspase-3 on TNF-α and/or 4-HC-induced apoptosis, we determined whether these apoptosis could be inhibited by caspase-3 inhibitor. TNF-α (50 ng/ml) and/or 4-HC (10 μM)-induced apoptosis was significantly attenuated by treatments with caspase-3 inhibitor (Fig. 5). These results also suggested that the apoptosis were mediated by the activation of caspase-3.

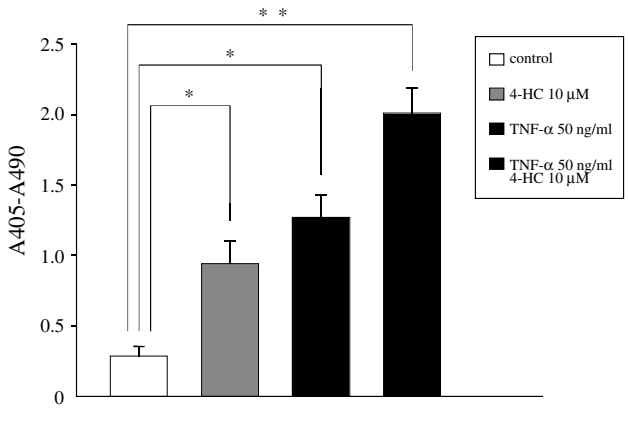

Fig. 3. Effects of TNF-α and 4-HC on DNA fragmentation in F-2. F-2 were incubated for 2 h in the presence or absence of TNF-α (50 ng/ml) and then cultured with 4-HC (10 μM) or vehicle for 24 h. The sandwich ELISA that can detect the enrichment of mono- or oligonucleosomes in cytoplasms of apoptotic cells was carried out as described in Section 2. The data are presented as means ± S.D. from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 1
Caspase-3 like protease (DEVDase) activation in F-2

	TNF-α (50 ng/ml)	4-HC (10 μM)
Caspase-3 activity (fold increase)	1.81 ± 0.17*	2.84 ± 0.13**

F-2 were incubated for 2 h with TNF-α (50 ng/ml), followed by 4-HC (10 μM) or vehicle for 24 h. Hydrolysis of DEVD-pNA substrate was measured at 405 nm. The data are means ± S.D. of three independent experiments.
$^{*}P < 0.05$ vs. control.
$^{**}P < 0.01$ vs. control.

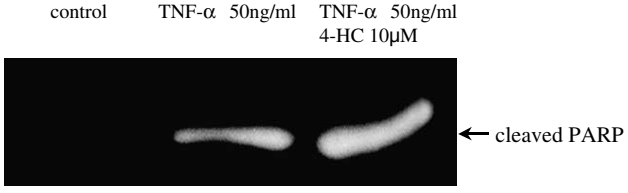

Fig. 4. Effects of TNF-α with or without 4-HC on the expression of the cleaved subunit of PARP in F-2. F-2 were incubated for 2 h in the presence of TNF-α (50 ng/ml) and then cultured with 4-HC (10 μM) or vehicle for 24 h. The cell lysates were analyzed by Western blotting with antibody to PARP.

3.4. The activation of caspase-8 was identified by the treatment of TNF-α, whereas the activation of caspase-9 was by 4-HC

We next examined whether "upstream" caspases such as caspase-8 and caspase-9 contributed to the apoptosis. IETDase activation was induced by TNF-α (50 ng/ml), but not by 4-HC (10 μM). In contrast, LEHDase activation was induced by 4-HC (10 μM), but not by TNF-α (50 ng/ml) (Table 2).

3.5. The intracellular expression of pro-apoptotic protein Bax was increased by the treatment of 4-HC

Further, we examined the expression levels of Bax, which is the major pro-apoptotic Bcl-2 protein, by Western blot analysis. Expression of Bax in the cells treated with TNF-α (50 ng/ml) was similar to the cells untreated, whereas it was elevated in the cells treated with 4-HC (Fig. 6).

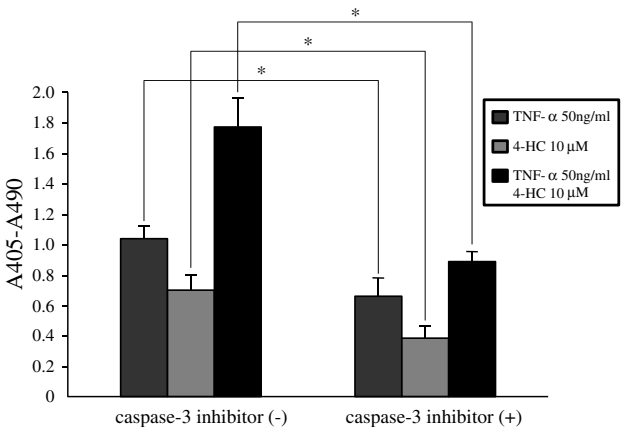

Fig. 5. Effects of caspase-3 inhibitors on TNF-α and/or 4-HC-induced apoptosis in F-2. F-2 were pretreated with Z-DEVD-FMK (10 μM) for 2 h and incubated for 2 h in the presence or the absence of TNF-α (50 ng/ml), followed by 4-HC (10 μM) or vehicle for 24 h. The data are presented as means ± S.D. from three independent experiments. $^{*}P < 0.05$.

Table 2
Caspase-8 like protease (IETDase) or caspase-9 like protease (LHEDase) activation in F-2

	TNF-α (50 ng/ml)	4-HC (10 μM)
Caspase-8 activity (fold increase)	1.74 ± 0.30*	1.25 ± 0.22
Caspase-9 activity (fold increase)	1.27 ± 0.17	1.88 ± 0.36*

F-2 were incubated for 24 h with TNF-α (50 ng/ml)or 4-HC (10 μM). Hydrolysis of IETD-pNA or LHED-pNA substrate was measured at 405 nm. The data are means ± S.D. of three independent experiments.
$^{*}P < 0.05$ vs. control.

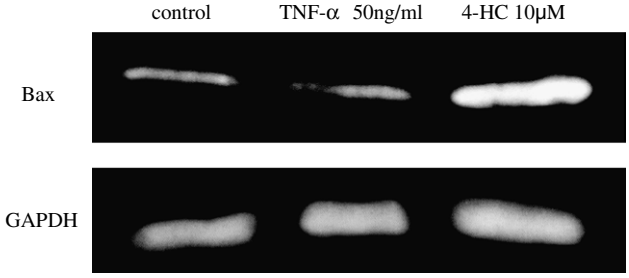

Fig. 6. Effects of TNF-α or 4-HC on the expression of Bax in F-2. F-2 were incubated with TNF-α (50 ng/ml)or 4-HC (10 μM) for 24 h. The cell lysates were analyzed by Western blotting with antibody to Bax.

4. Discussion

In our experiment, 4-HC (10 μM) inhibited the viability of murine dermal endothelial cells (F-2), whereas it did not exhibit significant effect on the viability of keratinocyte (PAM212) and fibroblast (NIH-3T3). The concentration used in this study in vitro, are equivalent to the physiological concentration after treatment of CPA in vivo [6]. Consistent with a recent report [7], these results demonstrated that endothelial cells were more susceptible to the effects of CPA than other cells. In addition, it was suggested that the inhibitions of cell viability were involved in apoptosis using DNA ladder assay and the cell death detection ELISA.

As the release of TNF-α is supposed to be elevated at inflammatory site, the ELISA assay was performed to clarify the

effect of 4-HC after TNF-α stimulation. The treatment with TNF-α (50 ng/ml) or 4-HC (10 μM) showed higher optical absorbance independently than no treatment, whereas TNF-α (50 ng/ml) and 4-HC (10 μM) have additional effect. Although it was reported that the stimulation with TNF-α alone did not induce DNA fragmentation of human umbilical vein endothelial cells (HUVEC) [8], our result indicated that TNF-α alone induced DNA fragmentation of F-2. The mechanisms of the differential sensitivity to TNF-α between HUVEC and F-2 is not currently unknown. However, one likely possibility is due to the expression pattern of anti-apoptotic proteins after TNF-α stimulation [9–11].

Recently, glucocorticoid was shown to suppress TNF-α-induced apoptosis in glomerular endothelial cells [12]. Indeed, this report is in accordance with our result that prednisolone inhibit TNF-α-induced DNA fragmentation in F-2 (data not shown). Although glucocorticoid is also effective for autoimmune diseases and systemic vasculitis [13], it is possible that the mechanism to endothelial cell is different from CPA.

Caspase-3 has been shown to play a pivotal role as a downstream member of the protease cascade, and several proteins were identified as caspase-3 substrates, such as PARP [14]. It was clarified that the treatment of 4-HC (10 μM) enhanced TNF-α (50 ng/ml)-induced caspase-3 like protease (DEVDase) activity and upregulated the expression of the cleaved subunit of PARP. Furthermore, TNF-α (50 ng/ml) and/or 4-HC (10 μM)-induced apoptosis of F-2 was significantly attenuated by treatments with caspase-3 inhibitor. These findings confirmed that caspase-3 activation was involved in the apoptosis induced by these reagents.

In an attempt to characterize the pathway, upstream of caspase-3, responsible for TNF-α or 4-HC-induced apoptosis of F-2, the activities of caspase-8 and caspase-9 were next investigated. Caspase-8 plays a important role in TNF-α-induced apoptosis by activating caspase-3 directly [15] or by triggering cytochrome *c* release from mitochondria through cleaved Bid, a pro-apoptotic Bcl-2 family member [16]. The cytochrome *c* binds to Apaf-1 and activates caspase-9, which in turn cleaves and activates caspase-3. In TNF-α-induced apoptosis of F-2, induction of caspase-8 like protease (IETDase) activity was observed, whereas caspase-9 like protease (LEHDase) activity was not changed. Then, it was suggested that main pathway of TNF-α induced apoptosis in F-2 was mitochondria-independent. In contrast, induction of caspase-9 like protease (IEHDase) activity was observed in 4-HC-induced apoptosis. CPA was reported to induce caspase-9 dependent apoptosis in other cells as well [17].

We further investigated the expression levels of Bax, one of the pro-apoptotic proteins, which plays a major role in initiating cytochrome *c* release [18]. We found that the expression was elevated in the cells treated with 4-HC, although the expression of Bcl-2 and Bcl-X_L, the anti-apoptotic proteins, which work to prevent Bax, thereby preserving cell survival, was not changed (data not shown).

In conclusion, we propose that there are different pathways to apoptotic cells, upstream of caspase-3, for TNF-α and CPA in murine vascular endothelial cells. If, as a result, CPA could easily induce the apoptosis of activated endothelial cells, it may be a suitable mechanism to limit inflammatory process. Our observations will provide new implications into the anti-inflammatory effects of CPA.

Acknowledgement: This work was supported by the grant from Funds for Research on Refractory Vasculitis of the Ministry of Health, Labor and Welfare and Segawa Research Grant.

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